

19



Europäisches Patentamt
European Patent Office
Office européen des brevets

11 Publication number:

**0 080 304
A1**

12

EUROPEAN PATENT APPLICATION

21 Application number: 82306025.6

51 Int. Cl.: C 12 Q 1/26, C 12 N 9/96

22 Date of filing: 11.11.82

30 Priority: 12.11.81 JP 181443/81

71 Applicant: WAKO PURE CHEMICAL INDUSTRIES, LTD.,
10, Doshomachi-3-chome, Higashi-ku Osaka (JP)

43 Date of publication of application: 01.06.83
Bulletin 83/22

72 Inventor: Miyashita, Yoshinobu,
14-17 Takadono-7-chome, Asahi-ku Osaka (JP)
Inventor: Satomura, Shinji, 5-25 Abikocho-1-chome,
Sumiyoshi-ku Osaka (JP)

84 Designated Contracting States: AT BE CH DE FR GB IT
LI LU NL SE

74 Representative: Baillie, Iain Cameron et al, c/o Langner
Parry Isartorplatz 5, D-8000 München 2 (DE)

54 Stabilization of oxidase.

57 An oxidase selected from the group consisting of glycerol-3-phosphate oxidase, choline oxidase and glucose oxidase can be stabilized by adding thereto an acidic amino acid or a salt thereof. The resulting stabilized composition can be used for quantitatively determining the content of glycerol-3-phosphate, choline, triglyceride, glucose, etc., in a biological fluid.

EP 0 080 304 A1

STABILIZATION OF OXIDASE

1 This invention relates to a process for stabilizing an oxidase and a composition containing the stabilized oxidase for use in clinical chemical examinations.

 Recently, clinical chemical examinations have
5 been developed remarkably as examination techniques for diagnosis of diseases and watching of the course of treatments. Particularly, the progress and spread of automatic chemical analysis equipments makes the examinations not only rapid and accurate but also more important
10 in hospital examinations. The automatic chemical analysis equipments require new type measuring reagents. That is, the reagents should complete the reaction in a short time at a mild temperature such as about 37°C in order to apply to such equipments. Thus, enzymatic measuring
15 methods using enzymes have been developed. At present, almost all blood components can be determined quantitatively by enzymatic methods.

 In such enzymatic methods, it becomes important to stabilize a reagent solution containing an enzyme for
20 a long period of time. For example, measurement of neutral fat (triglyceride) is an important test item, since it can be an important indication for finding lipids metabolism abnormal, diagnosis of diseases such as diabetes mellitus and judgement of treatment course.
25 For measuring triglyceride, glycerol-3-phosphate oxidase

1 is used. But glycerol-3-phosphate oxidase is not good
in stability, particularly in an aqueous solution.

It is also known that oxidases are generally
unstable. Stabilization of oxidases other than glycerol-
5 3-phosphate oxidase is also desirable. Examples of such
oxidases are choline oxidase, glucose oxidase, etc.

Main constituent of enzymes is proteins.

Enzymes show special enzymatic actions depending on space
structures of these proteins. But the space structures
10 vary by influences of various factors and thus enzymes
lose activities. Therefore, it is necessary to stabilize
the enzymes.

There are proposed many processes for stabiliz-
ing enzymes. One process is to add a substrate or a
15 coenzyme to an enzyme to be stabilized. In proteins
of enzymes, there are one or more local portions having
strain which is unstable from the viewpoint of energy.
Such portions often become active portions of enzymes.
When a substrate or a coenzyme is bonded to such
20 portions, such portions are stabilized from the viewpoint
of energy, which results in stabilizing the enzymes.

Another process is to add a SH protecting reagent to
enzymes. When an enzyme has an active portion having
a SH group, it is effective to add a SH protecting
25 reagent such as mercaptoethanol, dithiothreitol or the
like to such an enzyme. Further, non-specific stabilizers
are sometimes used. For example, inert proteins such as
albumin, glycerol, lactose, etc. are sometimes effective.

1 But, in the case of oxidases such as glycerol-3-phosphate oxidase, cholin oxidase, glucose oxidase, etc., such stabilizing methods or stabilizers as mentioned above are not effective at all.

5 Objects of this invention is to provide a process for stabilizing oxidases, a composition containing such a stabilized oxidase for use in clinical chemical examinations.

This invention provides a process for stabilizing an oxidase selected from the group consisting of glycerol-3-phosphate oxidase, choline oxidase and glucose oxidase which comprises adding an acidic amino acid or a salt thereof to said oxidase.

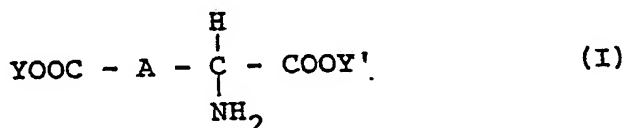
15 This invention also provides a composition for use in clinical chemical examinations comprising an oxidase selected from the group consisting of glycerol-3-phosphate oxidase, choline oxidase and glucose oxidase, and an acidic amino acid or a salt thereof.

In the attached drawings, Figs. 1 to 4 show relationships between residual activity of glycerol-3-phosphate oxidase and elapsed time with or without addition of a salt of acidic amino acid, Fig. 5 shows a relationship between residual activity of choline oxidase and elapsed time with or without addition of a salt of acidic amino acid, Fig. 6 shows a relationship between residual activity of glucose oxidase and elapsed time with or without addition of a salt of acidic amino acid, and Fig. 7 shows a relationship between residual

1 activity of cholesterol oxidase and elapsed time with
or without addition of a salt of acidic amino acid
(comparison).

It is a very important and surprising thing
5 that the addition of an acidic amino acid (aminodi-
carboxylic acid) or a salt thereof to an oxidase selected
from the group consisting of glycerol-3-phosphate oxidase,
choline oxidase and glucose oxidase makes the oxidase
stabilize without giving undesirable influences on the
10 measurement in clinical chemical examinations to be
conducted afterward.

The stabilizing agent usable in this invention
is an acidic amino acid or a salt thereof, preferably a
buffer solution-soluble salt thereof. Such a stabilizing
15 agent is preferably represented by the formula:



wherein A is a lower alkylene group preferably having
1 to 5 carbon atoms; and Y and Y' are independently
hydrogen, a NH_4 group or an alkali metal. Preferable
examples of the acidic amino acid of the formula (I)
20 are glutamic acid, aspartic acid, mono- or diammonium
salt of glutamic acid or aspartic acid, mono- or dialkali
metal salts of glutamic or aspartic acid such as sodium
glutamate, sodium aspartate, potassium glutamate,
potassium aspartate, etc. The use of alkali metal
25 salt of glutamic or aspartic acid is preferable

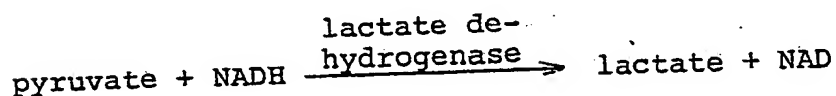
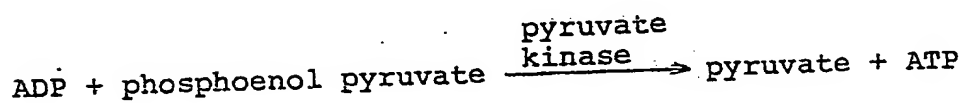
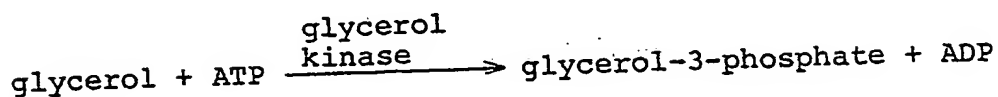
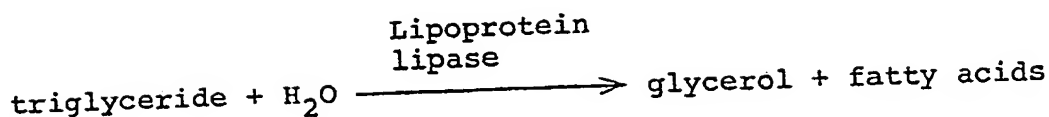
1 considering solubility. It is possible to use other
acidic amino acids such as α -aminoadipic acid, and the
like.

The acidic amino acid or a salt thereof is
5 added in an amount of 1 to 5% by weight to the aqueous
solution containing an oxidase to be stabilized. The
oxidase content in the aqueous solution changes depending
on the kinds of oxidases to be stabilized but usually
1 to 20 units/ml (U/ml) for glycerol-3-phosphate oxidase
10 and choline oxidase and 1 to 100 U/ml for glucose oxidase.
If the amount is too much, the stability of coloring in
the clinical chemical examination is damaged. Usually,
about 3% by weight is more preferable.

Oxidases to be stabilized by this invention are
15 glycerol-3-phosphate oxidase, choline oxidase and glucose
oxidase. Although these oxidases belong to flavin enzymes,
the stabilizing process of this invention is only
applicable to limited members of flavin enzymes. For
example, the process of this invention is not effective
20 for cholesterol oxidase which belongs to flavin enzymes.

Glycerol-3-phosphate oxidase is an oxidase
which can be obtained via culture and extraction from
strains of aerococcus or streptococcus, but it is
unstable particularly in the form an aqueous solution.

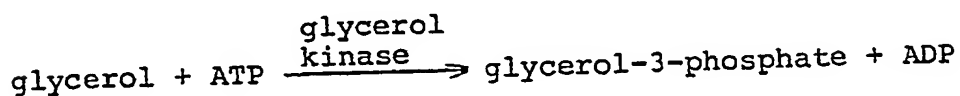
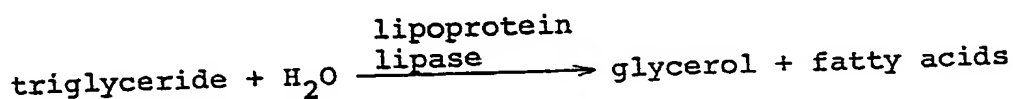
25 Glycerol-3-phosphate oxidase is used, for example,
for measuring triglyceride. Triglyceride (neutral fat)
was measured by using enzymes as follows:

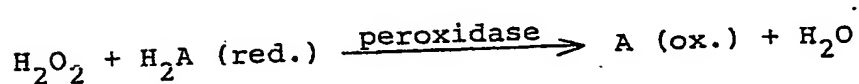
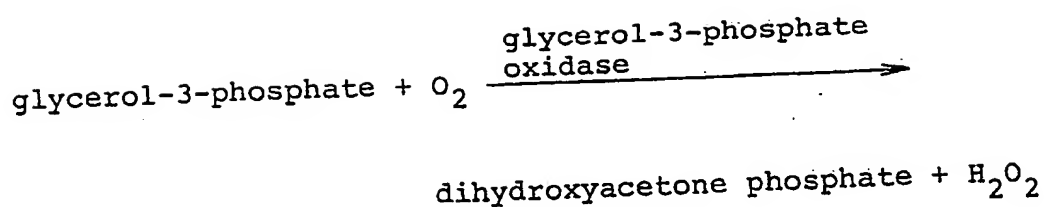


- 1 That is, by measuring a decrease in absorbance at 340 nm at which NADH shows a specific absorption, the content of triglyceride in a sample can be obtained. According to this method, since the absorbance at 340 nm which belongs
- 5 to ultraviolet region, it is necessary to use an ultraviolet spectrophotometer and further when serum is used as a sample, specimen blank gives great influence on measuring.

But, recently, a colorimetric method using

- 10 wavelengths in visible light region is developed in contrast to the above-mentioned method. Such a method uses glycerol-3-phosphate oxidase and can be represented by the following equations:

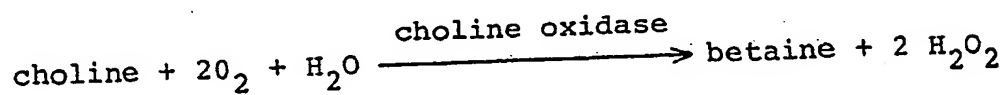




1 In the above-mentioned reaction equations, when
 an indicator which produces a color in a visible light
 region is used, it becomes possible to employ a colori-
 metric method in the visible light region while over-
 5 coming disadvantages of the old process mentioned above.
 Thus, to stabilize an aqueous solution of glycerol-3-
 phosphate oxidase becomes very important.

Choline oxidase is an oxidase which can be
 obtained from strains of arthrobacter or alcaligenes.

10 Choline oxidase accelerates the following
 reaction:

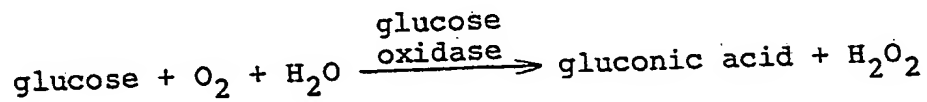


Therefore, in a system wherein choline is present or
 choline is produced, it becomes possible to employ a
 colorimetric method in the visible light region wherein
 15 H_2O_2 produced is measured. Further, as applications
 of choline oxidase to quantitative methods of living
 samples, it is possible to measure activity of choline
 esterase and to measure quantitatively the amount of

- 1 phospholipids wherein phospholipase D is combined and
liberated choline is measured.

Glucose oxidase is an oxidase which can be
obtained from strains of aspergillus and accelerates

- 5 the following reaction:



- Glucose oxidase have wide applications for
measuring living samples, for example, quantitative
determination of glucose in a body fluid, measuring of
activity of amylase, etc. In measuring activity of amy-
10 lase using starch as a substrate, glucose obtained by
decomposition of starch via glucoamylase is measured
quantitatively.

- In the next place, explanations will be given
to a stabilized reagent composition containing glycerol-
15 3-phosphate oxidase for measuring glycerol-3-phosphate
and the like. A reagent solution containing glycerol-
3-phosphate oxidase, peroxidase and an indicator, said
reagent solution per se being known for measuring glycerol-
3-phosphate quantitatively, can be stabilized by adding
20 an acidic amino acid or a salt thereof such as alkali
metal salt of aminodicarboxylic acid thereto. Thus, a
stabilized reagent composition for use in clinical
applications can be obtained, said composition containing
glycerol-3-phosphate oxidase, an alkali metal salt of
25 aminodicarboxylic acid as a stabilizer and a buffer

1 solution, and if necessary an indicator for colorimetric determination and one or more conventional additives.

In the same manner as mentioned above, stabilized reagent compositions comprising choline
5 oxidase or glucose oxidase, a stabilizer of the formula (I), and a buffer solution, and if necessary one or more conventional additives such as an indicator for colorimetric determination, and the like can be obtained.

In the case of measuring the content of tri-
10 glyceride in a living sample such as serum, etc., there can be used a reagent solution for measurement prepared by dissolving lipoprotein lipase, glycerol kinase, ATP, glycerol-3-phosphate oxidase, peroxidase and an indicator in a suitable buffer solution such as tris buffer
15 solution.

In the reaction using such a reagent composition, hydrogen peroxide (H_2O_2) is produced from glycerol-3-phosphate by the action of glycerol-3-phosphate oxidase. When an oxidizable color producing indicator is present
20 in such a case, said indicator produces the color by H_2O_2 in the presence of peroxidase.

As the oxidizable color producing indicator, there can be used o- or p-phenylenediamine, dianisidine, and the like indicators alone, or a combination indicator
25 such as 4-aminoantipyrine and phenol, a halophenol or an aniline derivative, etc.

As the buffer solution, there can be used any ones which can maintain the desired pH. Examples of such

1 buffer solutions are a tris buffer solution, Good buffer solution, phosphate buffer solution, and the like. The pH preferable for the reaction is near neutral value, and pH 7.5 is more preferable.

5 Further, as is clear from the reaction equations mentioned above, any substances such as triglyceride, glycerol, ATP, and glycerol-3-phosphate can be measured. But the most important reaction step among these reaction equations is the reaction using glycerol-3-phosphate
10 oxidase.

Glycerol-3-phosphate oxidase obtained from aerococcus viridans or streptococcus faecalis by a conventional process is very unstable in an aqueous solution and can only be used for a few hours after
15 dissolving.

But according to the stabilizing process of this invention, an aqueous solution of glycerol-3-phosphate oxidase can be stored at 5°C for one week stably by adding an aminodicarboxylic acid (acidic amino
20 acid) or a salt thereof and can be used for measurement during such a period. (See Figs. 1 to 4.)

In the same manner as mentioned above, an aqueous solution of choline oxidase can be stabilized and can be used for measurement after stored at 20°C for
25 one week, and an aqueous solution of glucose oxidase can be used for measurement after stored at 40°C for 3 days. (See Figs. 5 and 6.)

This invention is illustrated by way of the

1 following Examples.

Example 1

(1) Determination of Glycerol-3-phosphate

Reagent Solution

5 A reagent solution for measuring glycerol-3-phosphate was prepared by dissolving the following ingredients in 0.05 M tris buffer solution (pH 7.5):

	Glycerol-3-phosphate oxidase	5 U/ml
	Peroxidase	2.5 U/ml
10	p-Chlorophenol	0.07% by weight
	4-Aminoantipyrine	0.15 mg/ml
	Sodium glutamate	3.0% by weight

Measuring Operations

15 A sample (e.g. a biological fluid such as serum) in an amount of 0.02 ml was added to 3.0 ml of the reagent solution for measurement obtained by the above formulation. After mixing well, color was produced by warming at 37°C for 10 minutes. On the other hand, using 0.02 ml of distilled water, a reagent blank was prepared
20 in the same manner as mentioned above.

Absorbances at 505 nm were measured using the reagent blank as control. Absorbances of standard solutions prepared by dissolving certain amounts of glycerol-3-phosphate in various concentrations were also measured
25 in the same manner as mentioned above and the content of glycerol-3-phosphate in the sample was obtained by proportion calculations of the absorbances obtained.

1 (2) Stabilization of Aqueous Solution of Glycerol-3-phosphate Oxidase

 A 0.05 M tris buffer solution (pH 7.5) dissolv-
ing 0.07% by weight of p-chlorophenol, 5 U/ml of
5 glycerol-3-phosphate oxidase and 3.0% by weight of
sodium glutamate was maintained at 5°C and residual
activity of glycerol-3-phosphate oxidase was measured
with the lapse of time. For comparison, the same
composition as mentioned above except for not containing
10 sodium glutamate was also prepared and measured in the
same manner as mentioned above.

 The results are as shown in Fig. 1.

 When 3.0% by weight of sodium aspartate was
used in place of sodium glutamate, the results are as
15 shown in Fig. 2, which also shows the results of Fig. 1.

 When sodium glutamate was dissolved in a 0.05 M
tris buffer solution (pH 7.5) in various concentrations
(5%, 3%, 1%, and 0% by weight) together with 5 U/ml of
glycerol-3-phosphate oxidase and kept at 20°C, the
20 residual activity of glycerol-3-phosphate oxidase with
the lapse of time was as shown in Fig. 3.

 When sodium aspartate was dissolved in a 0.05 M
tris buffer solution (pH 7.5) in various concentrations
(3%, 1% and 0% by weight) together with 5 U/ml of
25 glycerol-3-phosphate oxidase and kept at 20°C, the
residual activity of glycerol-3-phosphate oxidase with
the lapse of time was as shown in Fig. 4.

1 Example 2

Determination of Triglyceride in a Living Sample

Reagent Solution

5 A reagent solution for measuring triglyceride was prepared by dissolving the following ingredients in 0.05 M tris buffer solution (pH 7.5):

	Lipoprotein lipase	40 U/ml
	Glycerol kinase	2.5 U/ml
	Glycerol-3-phosphate oxidase	5 U/ml
10	Peroxidase	2.5 U/ml
	Magnesium acetate	5 mmoles/l
	p-Chlorophenol	0.07% by weight
	4-Aminoantipyrine	0.15 mg/ml
	ATP	1 mg/ml
15	Sodium glutamate	3% by weight

Measuring Operations

A living sample or biological fluid (e.g. serum) in an amount of 0.02 ml was added to 3.0 ml of the reagent solution for measuring triglyceride and mixed well. Color was produced by warming at 37°C for 10 minutes. On the other hand, using 0.02 ml of distilled water, a reagent blank was prepared in the same manner as mentioned above.

Absorbances at 505 nm were measured using the reagent blank as control. Absorbances of standard solutions prepared by dissolving certain amounts of glycerol in various concentrations were also measured. The glycerol content was obtained by proportion

- 1 calculations, after which the triglyceride content was calculated by converting to the triglyceride amount.

Example 3

(1) Determination of Choline

5 Reagent Solution

A reagent solution for measuring choline was prepared by dissolving the following ingredients in a 0.05 M phosphate buffer solution (pH 7.6):

	Choline oxidase	2.5 U/ml
10	Peroxidase	1.0 U/ml
	4-Aminoantipyrine	0.015% by weight
	Phenol	0.1% by weight
	Sodium glutamate	3.0% by weight

Measuring Operations

- 15 A sample (e.g. a biological fluid such as serum) in an amount of 0.02 ml was added to 3.0 ml of the reagent solution for measuring choline and mixed well. Color was produced by warming at 37°C for 10 mintes. On the other hand, using 0.02 ml of distilled water,
20 a reagent blank was prepared in the same manner as mentioned above.

Absorbances at 505 nm were measured using the reagent blank as control. Absorbances of standard solutions prepared by dissolving certain amounts of
25 choline chloride in various concentrations were also measured. The choline content in the sample was obtained by proportion calculations of the absorbances obtained.

1 (2) Stabilization of Aqueous Solution of Choline Oxidase

A 0.05 M phosphate buffer solution (pH 7.6) dissolving 2.5 U/ml of choline oxidase and 0.1% by weight of phenol together with sodium glutamate (5%, 3%, 5 1% and 0% by weight) or sodium aspartate (3%, 1% and 0% by weight) was maintained at 20°C. The residual activity of choline oxidase was measured with the lapse of time and shown in Fig. 5.

Example 4

10 Measurement of Activity of Choline Esterase in Living Sample

Reagent Solution

(A) Substrate enzyme solution

A substrate enzyme solution was prepared by 15 dissolving the following ingredients in a 0.02 M phosphate buffer solution (pH 7.6):

Choline oxidase	2.5 U/ml
Peroxidase	1.0 U/ml
4-Aminoantipyrine	0.015% by weight
20 Choline benzoyl chloride	0.015% by weight
Phenol	0.2% by weight
Sodium glutamate	3.0% by weight

(B) Reaction stopper solution

A reaction stopper solution was prepared by 25 dissolving 100 mg of neostigmine methylsulfate in 100 ml of distilled water.

1 Measuring Operations

In a test tube, 2.0 ml of the substrate enzyme solution was placed and warmed at 37°C for 3 minutes in a constant temperature water bath. Subsequently, 0.02 ml of a sample (e.g. a biological fluid such as serum) was added to the test tube and mixed well. After warming at 37°C for just 5 minutes, 2.0 ml of the reaction stopper solution was added thereto. On the other hand, using 0.02 ml of distilled water, a reagent blank was prepared in the same manner as mentioned above.

Absorbances at 505 nm were measured using the reagent blank as control. Absorbances of serum having known activity values were measured in same manner as mentioned above and activity value of choline esterase in the sample was obtained by proportion calculations of the absorbances obtained.

Example 5

(1) Determination of Glucose

Reagent Solution

A reagent solution for measuring glucose was prepared by dissolving the following ingredients in a 0.2 M phosphate buffer (pH 7.4):

	Glucose oxidase	30 U/ml
	Mutarotase	0.1 U/ml
25	Peroxidase	1.0 U/ml
	4-Aminoantipyrine	0.1% by weight
	Phenol	0.1% by weight
	Sodium aspartate	5.0% by weight

1 Measuring Operations

A sample (e.g. a biological fluid such as serum) in an amount of 0.02 ml was added to 3.0 ml of the reagent solution for measuring glucose and mixed well.

5 Color was produced by warming at 37°C for 10 minutes.

On the other hand, using 0.02 ml of distilled water, a reagent blank was prepared in the same manner as mentioned above.

Absorbances at 505 nm were measured using the reagent blank as control. Absorbances of standard solutions prepared by dissolving certain amounts of glucose in various concentration were also measured. The glucose content in the sample was obtained by proportion calculations of the absorbances obtained.

15 (2) Stabilization of Aqueous Solution of Glucose Oxidase

A 0.2 M phosphate buffer solution (pH 7.4) dissolving 30 U/ml of glucose oxidase and 0.1% by weight of phenol together with sodium glutamate (5%, 3%, 1% and 0% by weight) or sodium aspartate (5%, 3%, 1% and 0% by weight) was maintained at 40°C. The residual activity of glucose oxidase was measured with the lapse of time and shown in Fig. 6.

Comparative Example 1

5 (1) Determination of Cholesterol

Reagent Solution

A reagent solution for measuring cholesterol was prepared by dissolving the following ingredients in

1 a 0.1 M phosphate buffer solution (pH 7.0):

Cholesterol oxidase	0.2 U/ml
Peroxidase	1.0 U/ml
4-Aminoantipyrine	0.015% by weight
5 Phenol	0.1% by weight

Measuring Operations

A sample (e.g. a biological fluid such as serum) in an amount of 0.02 ml was added to 3.0 ml of the reagent solution for measuring cholesterol and
10 mixed well. Color was produced by warming at 37°C for 10 minutes. On the other hand, using 0.02 ml of distilled water, a reagent blank was prepared in the same manner as mentioned above.

Absorbances at 505 nm were measured using
15 the reagent blank as control. Absorbances of standard solutions prepared by dissolving certain amounts of cholesterol in isopropyl alcohol in various concentrations were also measured. The cholesterol content in the sample was obtained by proportion calculations of
20 the absorbances obtained. The same results were also obtained when sodium aspartate or sodium glutamate (3% by weight) was added to the reagent solution.

(2) Stabilization of Aqueous Solution of Cholesterol Oxidase

25 A 0.1 M phosphate buffer solution (pH 7.0) dissolving 0.2 U/ml of cholesterol oxidase and 0.1% by weight of phenol together with sodium glutamate (3% by weight) or sodium aspartate (3% by weight) or

1 without sodium glutamate or sodium aspartate was maintained at 20°C. The residual activity of cholesterol oxidase was measured with the lapse of time and shown in Fig. 7.

5 As shown in Fig. 7, there is shown no stabilizing effect of sodium aspartate or sodium glutamate in the case of aqueous solution of cholesterol oxidase.

WHAT IS CLAIMED IS:

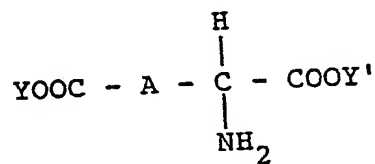
1. A process for stabilizing an oxidase which comprises adding an acidic amino acid or a salt thereof to an oxidase selected from the group consisting of glycerol-3-phosphate oxidase, choline oxidase and glucose oxidase.

2. A process according to Claim 1, wherein the oxidase is glycerol-3-phosphate oxidase.

3. A process according to Claim 1, wherein the oxidase is choline oxidase.

4. A process according to Claim 1, wherein the acidic amino acid or a salt thereof is a buffer solution-soluble salt of acidic amino acid.

5. A process according to Claim 1, wherein the acidic amino acid or a salt thereof is represented by the formula:



wherein A is a lower alkylene group having 1 to 5 carbon atoms; and Y and Y' are independently hydrogen, a NH_4 group or an alkali metal.

6. A process according to Claim 1, wherein the acidic amino acid or a salt thereof is sodium glutamate.

7. A process according to Claim 1, wherein the acidic amino acid or a salt thereof is sodium aspartate.

8. A process according to Claim 1, wherein the acidic amino acid or a salt thereof is added in an amount of 1 to 5% by weight to an aqueous solution containing the oxidase to be stabilized.

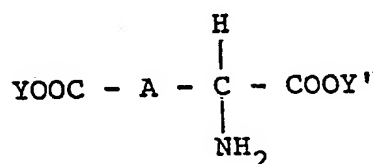
9. A stabilized composition comprising
a buffer solution,
an oxidase selected from the group consisting of glycerol-3-phosphate oxidase, choline oxidase and glucose oxidase, and

an acidic amino acid or a salt thereof.

10. A composition according to Claim 9, wherein the oxidase is glycerol-3-phosphate oxidase.

11. A composition according to Claim 9, wherein the oxidase is choline oxidase.

12. A stabilized composition according to Claim 9, wherein the acidic amino acid or a salt thereof is represented by the formula:



wherein A is a lower alkylene group having 1 to 5 carbon atoms; and Y and Y' are independently hydrogen, a NH_4 group or an alkali metal.

13. A composition according to Claim 9, which further comprises a color producing reagent.

14. A composition according to Claim 9, wherein

the acidic amino acid or a salt thereof is sodium glutamate.

15. A composition according to Claim 9, wherein the acidic amino acid or a salt thereof is sodium aspartate.

16. A diagnostic test kit for the determination of hydrogen peroxide which comprises

- a) a container having a composition comprising a buffer solution,

an oxidase selected from the group consisting of glycerol-3-phosphate oxidase, choline oxidase and glucose oxidase, and

an acidic amino acid or a salt thereof, and

- b) a container having a composition comprising a color producing reagent, and a solvent for the color producing reagent.

17. A process for using a composition comprising glycerol-3-phosphate oxidase,

a buffer solution,

an acidic amino acid or a salt thereof,

peroxidase, and

4-aminoantipyrine,

for quantitatively determining the glycerol-3-phosphate content in a biological fluid.

18. A process according to Claim 17, wherein the acidic amino acid or a salt thereof is an alkali metal salt of acidic amino acid.

19. A process according to Claim 18, wherein the

alkali metal salt of acidic amino acid is sodium aspartate or sodium glutamate.

20. A process for using a composition comprising

a lipase,

glycerol kinase,

glycerol-3-phosphate oxidase,

peroxidase,

4-aminoantipyrine,

adenosine triphosphate,

an acidic amino acid or a salt thereof, and

a buffer solution,

for quantitatively determining the triglyceride content in a biological fluid.

21. A process according to Claim 20, wherein the acidic amino acid or a salt thereof is an alkali metal salt of acidic amino acid.

22. A process according to Claim 21, wherein the alkali metal salt of acidic amino acid is sodium aspartate or sodium glutamate.

23. A process for using a composition comprising

choline oxidase,

peroxidase,

4-aminoantipyrine,

an acidic amino acid or a salt thereof, and

a buffer solution,

for quantitatively determining the choline content in a biological fluid.

24. A process according to Claim 23, wherein the

acidic amino acid or a salt thereof is an alkali metal salt of acidic amino acid.

25. A process according to Claim 24, wherein the alkali metal salt of acidic amino acid is sodium aspartate or sodium glutamate.

26. A process for using a composition comprising
glucose oxidase,
peroxidase,
mutarotase,
4-aminoantipyrine,
an acidic amino acid or a salt thereof, and
a buffer solution,

for quantitatively determining the glucose content in a biological fluid.

27. A process according to Claim 26, wherein the acidic amino acid or a salt thereof is an alkali metal salt of acidic amino acid.

28. A process according to Claim 27, wherein the alkali metal salt of acidic amino acid is sodium aspartate or sodium glutamate.

29. A process for using a composition comprising
choline oxidase,
peroxidase,
4-aminoantipyrine,
choline benzoyl chloride,
an acidic amino acid or a salt thereof, and
a buffer solution,

for measuring activity of choline esterase in a biological

fluid.

30. A process according to Claim 29, wherein the acidic amino acid or a salt thereof is an alkali metal salt of acidic amino acid.

31. A process according to Claim 30, wherein the alkali metal salt of acidic amino acid is sodium aspartate or sodium glutamate.

1/5

FIG. 1

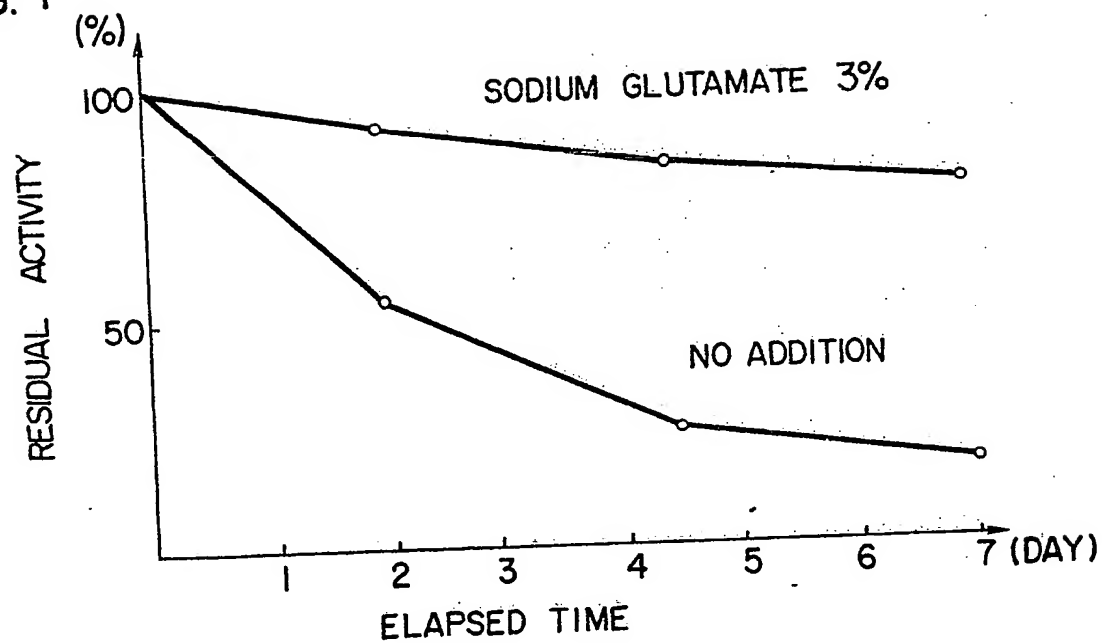


FIG. 2

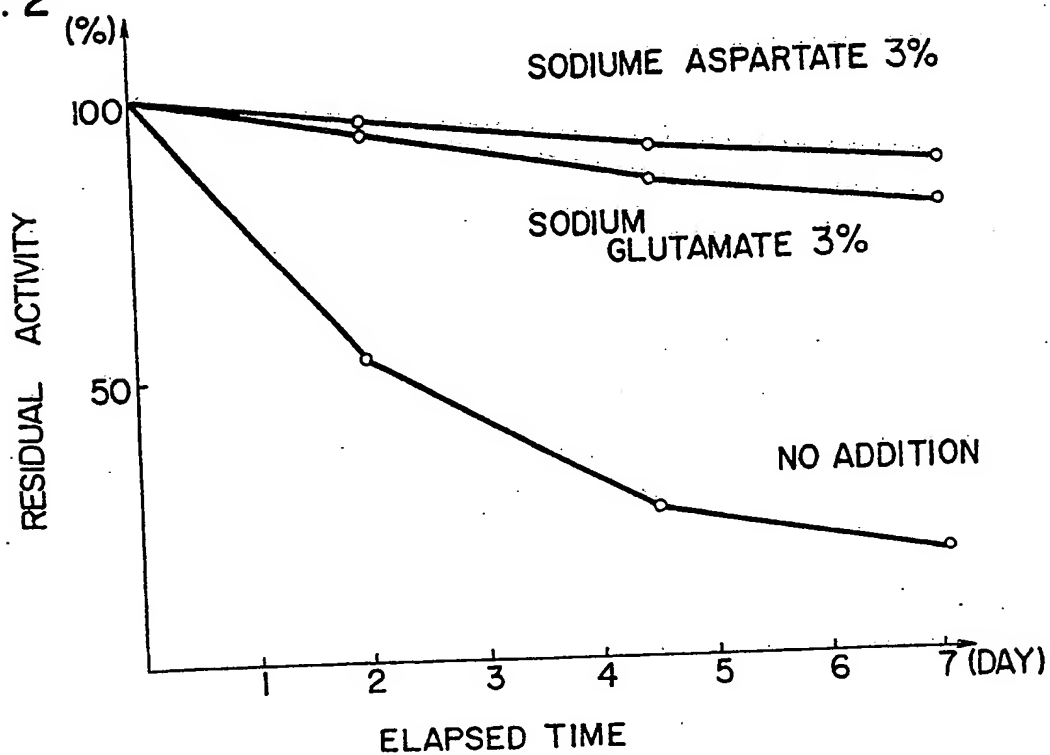


FIG. 3

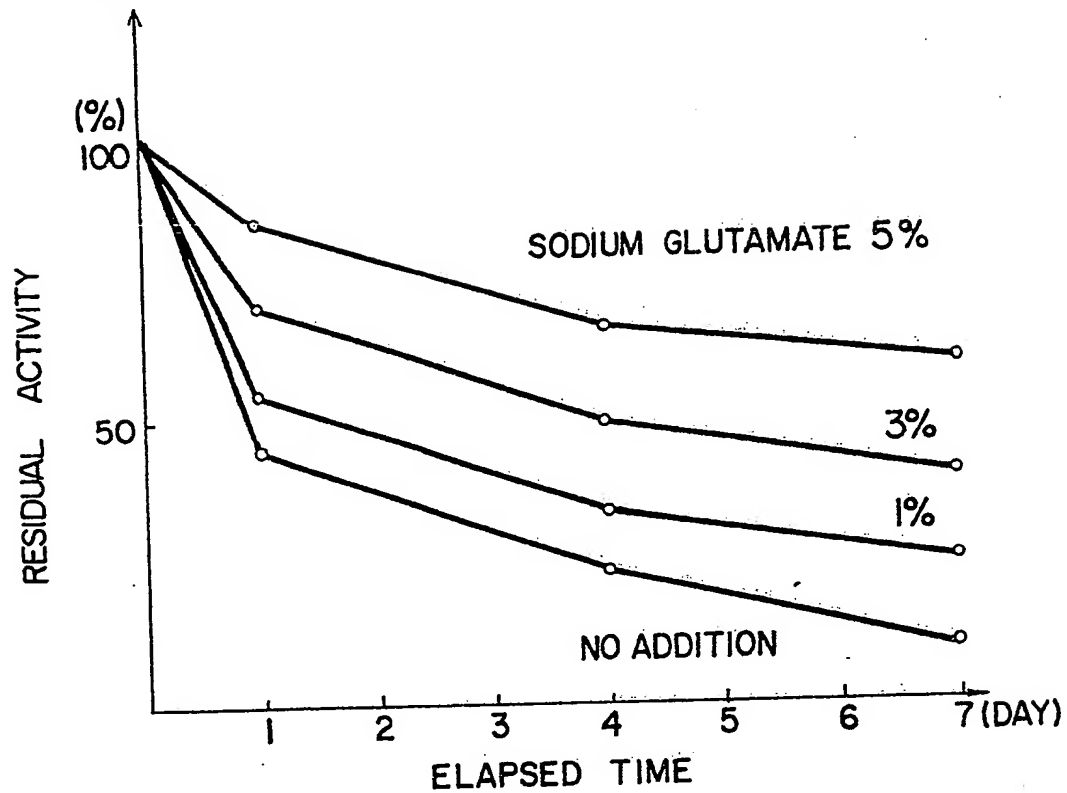


FIG. 4

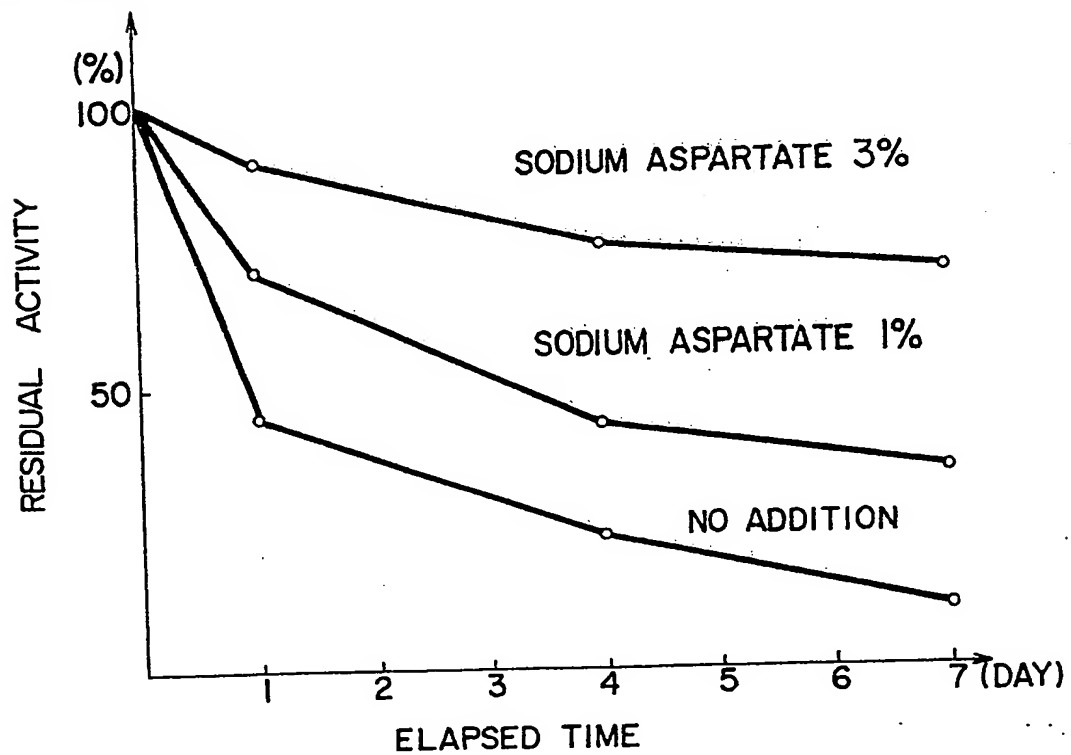


FIG. 5

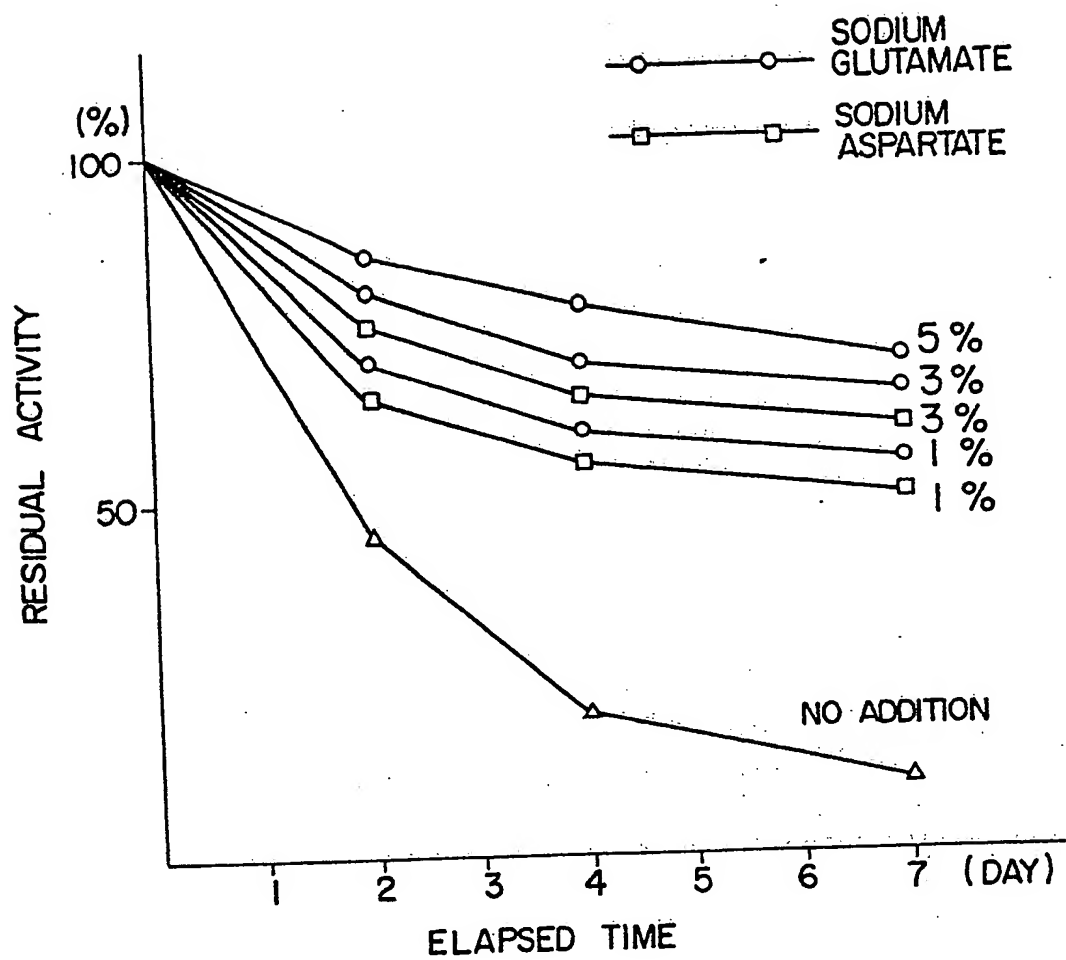


FIG. 6

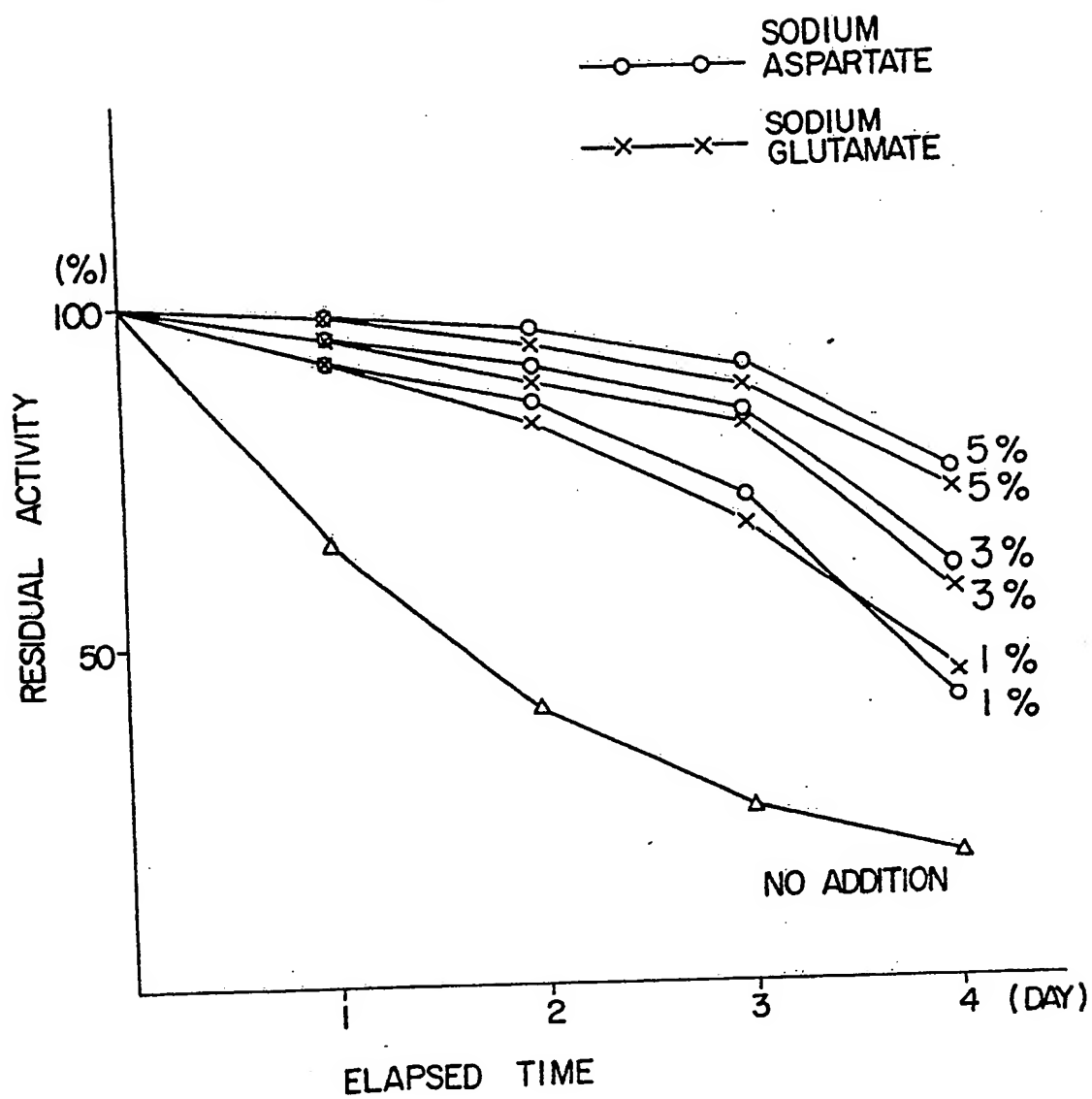
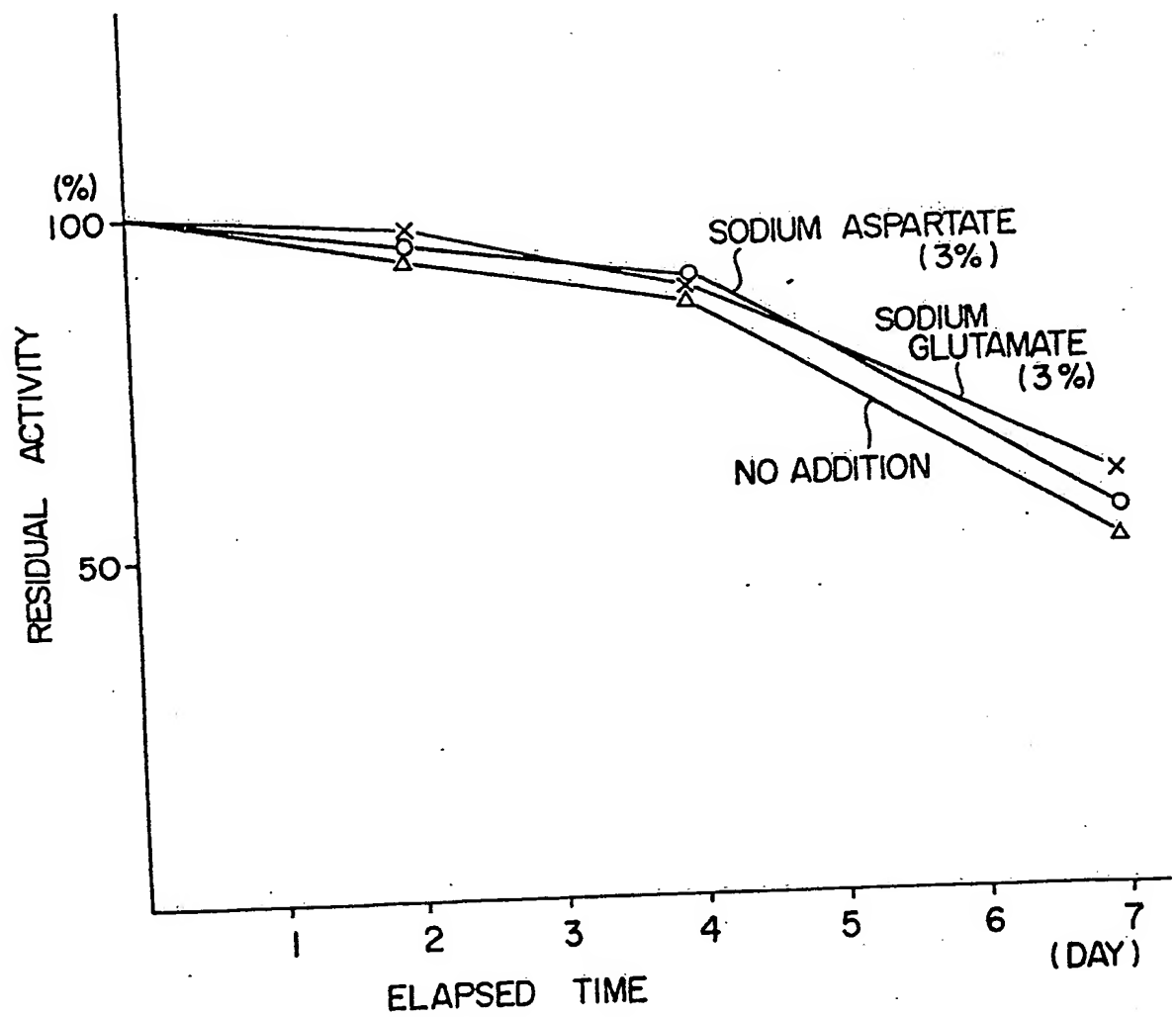


FIG. 7





European Patent
Office

EUROPEAN SEARCH REPORT

0080304
Application number

EP 82 30 6025

DOCUMENTS CONSIDERED TO BE RELEVANT			
Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int. Cl. 7)
Y	--- CHEMICAL ABSTRACTS, vol. 93, no. 7, August 1980, page 785, col. 1, no. 68683a, Columbus Ohio (USA); & JP - A - 80 34 001 (NODA INSTITUTE FOR SCIENTIFIC RESEARCH) (10-03-1980) *Abstract*	1,5-7 9,12, 14,15 18,19 21,22 24,25 27,28	C 12 Q 1/26 C 12 N 9/96
Y	CHEMICAL ABSTRACTS	30,31	
Y	--- EP-A-0 009 222 (AMERICAN MONITOR CORP.) *Page 21, lines 20-29; page 22, lines 1-7; pages 32-34*	4,5,7	
Y	--- US-A-3 964 974 (D.BENAUCH et al.) *Column 1, lines 42-54; column 4, lines 1-58; claim 1*	26	TECHNICAL FIELDS SEARCHED (Int. Cl. 7) C 12 Q C 12 N
Y	--- GB-A-2 033 082 (ISTITUTO SIEROTERAPICO E VACCINOGENO TOSCANO "SCLAVO") *Page 2, line 30 - page 3, line 51; claim 1*	1,9,13 ,16,26	
--- -/-			
The present search report has been drawn up for all claims			
Place of search THE HAGUE		Date of completion of the search 11-02-1983	Examiner GRIFFITH G.
CATEGORY OF CITED DOCUMENTS		T : theory or principle underlying the invention E : earlier patent document, but published on, or after the filing date D : document cited in the application L : document cited for other reasons & : member of the same patent family, corresponding document	
X : particularly relevant if taken alone Y : particularly relevant if combined with another document of the same category A : technological background O : non-written disclosure P : intermediate document			



European Patent
Office

EUROPEAN SEARCH REPORT

0080304
Application number

EP 82 30 6025

Page 2

DOCUMENTS CONSIDERED TO BE RELEVANT			
Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int. Cl. *)
Y	US-A-4 275 161 (H.MISAKI et al.) *Column 2, lines 34-64; example 3*	1,2,9 10,13 16,17	
Y	US-A-4 135 980 (S.IKUTA et al.) *Column 5, lines 6-68; column 7, lines 6-34; column 9, lines 32-48; column 10, lines 26-57; examples 2,5; claims 1,7,8,14,15,16,19,21*	1,3,9 11,13 16,23 29	
Y	GB-A-2 018 988 (KYOWA HAKKO KOGYO KABUSHIKI KAISHA) *Page 4, line 43 - page 7, line 21; examples 1-4; claims 1-4,11-14*	29	
Y	US-A-4 241 178 (T.W.ESDERS et al.) *Column 7 - column 11; claims 1,3-6,17-20,21,23,24,29,40-42,44,50,51*	16,20	
Y	EP-A-0 010 296 (KYOWA HAKKO KOGYO CO. LTD.) *Pages 3-5; examples 3,4; claims 1-5*	20	
The present search report has been drawn up for all claims			
Place of search THE HAGUE		Date of completion of the search 11-02-1983	Examiner GRIFFITH G.
CATEGORY OF CITED DOCUMENTS X : particularly relevant if taken alone Y : particularly relevant if combined with another document of the same category A : technological background O : non-written disclosure P : intermediate document T : theory or principle underlying the invention E : earlier patent document, but published on, or after the filing date D : document cited in the application L : document cited for other reasons & : member of the same patent family, corresponding document			



European Patent
Office

EUROPEAN SEARCH REPORT

0080304

Application number

EP 82 30 6025

Page 3

DOCUMENTS CONSIDERED TO BE RELEVANT			CLASSIFICATION OF THE APPLICATION (Int. Cl. ³)
Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	
A	EP-A-0 027 236 (MILES LABORATORIES INC.)		

A	US-A-3 096 251 (A.L. BABSON)		

			TECHNICAL FIELDS SEARCHED (Int. Cl. ³)
The present search report has been drawn up for all claims			
Place of search THE HAGUE		Date of completion of the search 11-02-1983	Examiner GRIFFITH G.
CATEGORY OF CITED DOCUMENTS			
X : particularly relevant if taken alone Y : particularly relevant if combined with another document of the same category A : technological background O : non-written disclosure P : intermediate document			
T : theory or principle underlying the invention E : earlier patent document, but published on, or after the filing date D : document cited in the application L : document cited for other reasons & : member of the same patent family, corresponding document			